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## **Sulfation of glucuronic acid in the linkage tetrasaccharide by HNK-1 sulfotransferase is an inhibitory signal for the expression of a chondroitin sulfate chain on thrombomodulin**

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### **Abstract**

HNK-1 (human natural killer-1) carbohydrate epitope (HSO<sub>3</sub>-3GlcAβ1-3Galβ1-4GlcNAc-) recognized by a HNK-1 monoclonal antibody is highly expressed in the nervous system and biosynthesized by a glucuronyltransferase (GlcAT-P or GlcAT-S), and sulfotransferase (HNK-1ST). A similar oligosaccharide (HSO<sub>3</sub>-3GlcAβ1-3Galβ1-3Galβ1-4Xyl) also recognized by the HNK-1 antibody had been found in a glycosaminoglycan (GAG)-protein linkage region of α-thrombomodulin (TM) from human urine. However, which sulfotransferase is involved in sulfation of the terminal GlcA in the GAG-protein linkage region remains unclear. In this study, using CHO-K1 cells in which neither GlcAT-P nor GlcAT-S is endogenously expressed, we found that HNK-1ST has the ability to produce HNK-1 immnoreactivity on α-TM. We also demonstrated that HNK-1ST caused the suppression of chondroitin sulfate (CS) synthesis on TM and a reduction of its anti-coagulant activity. Moreover, using an *in vitro* enzyme assay system, the HNK-1-positive TM was found not to be utilized as a substrate for CS-polymerizing enzymes (chondroitin synthase (ChSy) and chondroitin polymerizing factor (ChPF)). These results suggest that HNK-1ST is involved in 3-*O*-sulfation of the terminal GlcA of the linkage tetrasaccharide which acts as an inhibitory signal for the initiation of CS biosynthesis on TM.

**Key words:** HNK-1 carbohydrate epitope, thrombomodulin, HNK-1ST, GAG-protein linkage region, anti-coagulant activity

## 1. Introduction

Proteoglycans (PGs) ubiquitously expressed on the cell surface and in the extracellular matrix, are physiologically essential macromolecules modified by glycosaminoglycan (GAG) sidechains including chondroitin sulfate (CS) and heparan sulfate (HS). GAGs have a linear polysaccharide structure comprised of repeating disaccharide units,  $[(-4\text{GlcA}\beta 1-3\text{GalNAc}\beta 1-)]_n$  for CS and  $[(-4\text{GlcA}\beta 1-4\text{GlcNAc}\alpha 1-)]_n$  for HS [1]. The polymerization of GAGs is triggered by the first transfer reaction of GalNAc or GlcNAc to the non-reducing terminus of the GAG-protein linkage tetrasaccharide,  $\text{GlcA}\beta 1-3\text{Gal}\beta 1-3\text{Gal}\beta 1-4\text{Xyl-O-Ser}$  [1]. This unique tetrasaccharide is generated by xylosyltransferase (XylT), galactosyltransferase (GalT)-I, GalT-II, and glucuronyltransferase I (GlcAT-I) [1-6]. GAGs attached to a protein core are further processed by modifications such as sulfation and epimerization to various degrees dependent on tissue type or developmental stage, generating fully functional PGs [7, 8].

Thrombomodulin (TM), a cell surface PG, is a critical mediator of endothelial anti-coagulant defenses occurring as both a CS-PG ( $\beta$ -TM) and a protein ( $\alpha$ -TM) unsubstituted by CS, hence its description as a “part-time” PG [9]. Studies on the anti-coagulant effects of TM demonstrated that it has cofactor activity for thrombin-dependent activation of protein C, direct inhibition of fibrinogen cleavage by thrombin, and indirect enhancement of the association of antithrombin III with thrombin [10]. We previously demonstrated that a recombinant human  $\alpha$ -TM expressed in CHO-K1 cells bore a truncated un-modified tetrasaccharide linkage structure [11]. Comparative studies on the anti-coagulant effects of  $\alpha$ -TM and  $\beta$ -TM revealed that the CS chain of TM plays an important role in regulating the anti-coagulative functions [12]. Thus, it is important to investigate a possible biosynthetic mechanism for part-time PGs.

Human natural killer-1 (HNK-1), a neural carbohydrate epitope abundantly expressed during the brain development, is required for the acquisition of higher-order brain functions including synaptic plasticity [13-15]. A structural feature of this epitope is the terminal sulfated glucuronic acid. Therefore, glucuronyltransferase (GlcAT-P or GlcAT-S) and HNK-1 sulfotransferase (HNK-1ST) are key enzymes for HNK-1 biosynthesis. We demonstrated that GlcAT-P (also GlcAT-S) and HNK-1ST specifically interact and form an enzyme complex effective of HNK-1 biosynthesis [16]. Intriguingly, we also found that HNK-1ST interacts with GlcAT-I, implying a physiological role for this association [16]. In addition, 3-O-sulfation of the terminal GlcA of the linkage tetrasaccharide was identified in  $\alpha$ -TM isolated from human urine and this structure was recognized by a HNK-1 monoclonal antibody (mAb) [17]. These findings,

led us to the hypothesis that HNK-1ST is the sulfotransferase responsible for the terminal 3-*O*-sulfation of the linkage region. In this regard, recent studies identified various modifications of the linkage tetrasaccharide and revealed their effects on GAG biosynthesis, providing important clues as to the biosynthetic mechanism of GAG. For instance, 2-*O*-phosphorylation of xylose positively affects GlcAT-I activity, facilitating the linkage tetrasaccharide synthesis [18]. FAM20B was identified as a kinase responsible for the 2-*O*-phosphorylation of xylose and shown to modulate the number of GAG chains [19]. Moreover, during the preparation of this manuscript, Hashiguchi *et al.* demonstrated the sulfotransferase activity of HNK-1ST toward the GlcA of the GAG-linkage region with an *in vitro* enzymatic assay system using a peptide containing the tetrasaccharide [20]. However, the biological significance of the sulfate group on GlcA remains unclear.

In this study, we explored the involvement of HNK-1ST in the modification of the protein-linkage region and regulation of GAG assembly. Using a soluble form of recombinant TM, we found that HNK-1ST actually sulfated the linkage region and induced immunoreactivity with a HNK-1 mAb. HNK-1ST had the ability to suppress the GAG-bearing form of TM ( $\beta$ -TM) and anti-coagulant activity of TM. Moreover, CS chain polymerization reaction was not observed on the HNK-1-positive TM, suggesting the terminal 3-*O*-sulfation of the linkage tetrasaccharide to act as an inhibitory signal for the initiation of GAG biosynthesis.

## 2. Materials and methods

### 2.1. Materials

HNK-1 mAb was purchased from the American Type Culture Collection. The mouse anti-EGFP and anti-myc mAbs were from Clontech and Millipore, respectively. The HRP-conjugated anti-mouse IgG and anti-mouse IgM were obtained from Invitrogen. The nickel-nitrilotriacetic acid (Ni-NTA)-conjugated agarose was from Qiagen. Chondroitinase ABC and peptide *N*-glycosidase F were purchased from Seikagaku Corp., Tokyo, Japan and Roche Applied Science, respectively. UDP-[<sup>3</sup>H]GalNAc (10 Ci/mmol) was from NEN Life Science Products. Unlabeled UDP-GlcA and UDP-GalNAc were obtained from Sigma (St Louis, MO, USA).

### 2.2. cDNA construction

The cDNA fragment encoding TM was amplified from a human leukocyte cDNA library (Clontech) as a template with the primers CGGGATCCGGTAACATGCTTGGGGTCCTG containing a *Bam*HI site and CCAAGCTTTCGAATGCACGAGCCCCACGG containing a *Hind*III site. The PCR fragments were subcloned into pcDNA3.1(–) myc/His (Invitrogen) (sTM-myc). The expression plasmid for EGFP-tagged rat HNK-1ST (ST-EGFP) was described previously [16]. The plasmid encoding the R189A mutant of HNK-1ST (R189A-EGFP) was constructed from ST-EGFP, using a QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene) with the primers CAAGTTTTTTTATTGTGGCGGATCCCTTTGAAAGACTGATCTCTGC and GCAGAGATCAGTCTTTCAAAGGGATCCGCCACAATAAAAACTTG.

### 2.3. Cell culture and transfection

CHO-K1 cells were maintained in  $\alpha$ -minimum essential medium with 10% fetal bovine serum (FBS) in 5% CO<sub>2</sub> at 37°C. The cells were grown overnight and transfected with cDNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The culture medium was replaced with serum-free OPTI-MEM I (Invitrogen) 5 h after transfection, and the cells were cultured another 2 days.

### 2.4. Western blot analysis

Proteins solubilized in Laemmli sample buffer were separated by SDS-PAGE using 7% polyacrylamide gels and then transferred to nitrocellulose membranes. After blocking with 5%

nonfat dry milk in phosphate-buffered saline containing 0.05% Tween 20, the membranes were incubated with primary antibodies, followed by HRP-conjugated secondary antibodies. Protein bands were detected with Super Signal West Pico chemiluminescence reagent (Thermo Scientific) using a LAS-3000 Luminoimage Analyzer (FUJIFILM).

## 2.5. Purification of recombinant sTM-myc

CHO-K1 cells plated on 175 cm<sup>2</sup> tissue culture flasks were transfected with sTM-myc with or without ST-EGFP using the Lipofectamine 2000 transfection reagent. After 5 h, the culture medium was replaced with serum-free ASF104 medium (Ajinomoto), and the cells were cultured for another 3 days. Then, the culture medium containing secreted proteins was applied to a HisTrap TM HP column (GE Healthcare). Unbound proteins were washed out with more than 10 column volumes of phosphate buffer (pH 7.4) containing 300 mM NaCl and 20 mM imidazole. Bound proteins were eluted with phosphate buffer (pH 7.4) containing 300 mM NaCl and 500 mM imidazole. Then, the eluted materials were dialyzed with 20 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl.

## 2.6. Measurement of the anti-coagulant activity of TM

The anti-coagulant activity of TM was determined by its ability to inhibit thrombin clotting activity with fibrinogen (Sigma) as described [10, 21]. Fibrinogen clotting was monitored visually. Thrombin (18 nM, Sigma) was incubated for 1 min at 37°C with TM, chondroitinase ABC-treated TM, or TM+HNK-1ST at various molar ratios. Fibrinogen (2.0 mg/ml, final concentration) was then added and the clotting time was determined. The assays were performed in 0.02 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl and 1 mg/ml bovine serum albumin.

## 2.7. CS polymerization assay

The ChSy-1 and ChPF expression plasmids (3.0 µg each) were co-transfected into COS-1 cells on 100-mm plates using FuGENE<sup>TM</sup> 6 (Roche Molecular Biochemicals, Tokyo, Japan) as described previously [22]. Two days after transfection, 1 ml of the culture medium was collected and incubated with 10 µl of IgG-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4°C. The beads recovered by centrifugation were washed with and resuspended in the assay buffer, and then tested for GalNAc transferase activity, as described [23]. Polymerization reactions using TM or TM+HNK-1ST as acceptors were co-incubated in reaction mixtures

containing the following constituents in a total volume of 50  $\mu$ l: 0.1 nmol TM or TM+HNK-1ST, 0.25 mM UDP-[ $^3$ H]GalNAc ( $5 \times 10^5$  dpm), 0.25 mM UDP-GlcA, 100 mM MES buffer, pH 6.5, 10 mM  $\text{MnCl}_2$ , and 10  $\mu$ l of the resuspended beads. The mixtures were incubated at 37°C overnight. Products of the polymerization reactions were isolated by gel filtration using a syringe column packed with Sephadex G-25 (superfine) [24]. The flow-through fractions were collected, and radioactivity was measured by liquid scintillation spectrophotometry.

### 3. Results and discussion

#### 3.1. HNK-1ST suppresses CS modification of TM

To investigate the involvement of HNK-1ST in the modification of the protein-linkage region in TM, a soluble form of myc/His-tagged TM (sTM-myc) was transiently expressed in CHO-K1 cells with or without EGFP-tagged HNK-1ST (ST-EGFP). Although TM is an integral membrane protein, sTM-myc is designed to be secreted into the culture medium by truncating the transmembrane domain. The secreted sTM-myc was pulled down from the culture medium with nickel-nitrilotriacetic acid (Ni-NTA)-conjugated agarose beads, and subjected to Western blotting. When sTM-myc was expressed alone, both TM glycoforms, the CS chain-bearing form ( $\beta$ -TM) and non-bearing form ( $\alpha$ -TM), were detected by anti-myc antibody (Fig. 1A). After digestion with chondroitinase ABC (CSase ABC), the upper smear ( $\beta$ -TM) disappeared and converged with the 100 kDa band ( $\alpha$ -TM) (Fig.1B), indicating that sTM-myc was actually modified by the CS chain in CHO-K1 cells. Interestingly, however, with the co-expression of sTM-myc and ST-EGFP,  $\beta$ -TM production was markedly reduced, and conversely, the amount of  $\alpha$ -TM increased, indicating that most TM remains as a CS chain-lacking  $\alpha$ -form in the presence of HNK-1ST (Fig. 1A). Moreover, HNK-1 immunoreactivity was detected on the  $\alpha$ -form of sTM-myc co-expressed with ST-EGFP (Fig. 1A). Since neither GlcAT-P nor GlcAT-S is endogenously expressed in CHO-K1 cells, the HNK-1 epitope might be synthesized by GlcAT-I and HNK-1ST. To further characterize the HNK-1 epitope on sTM-myc, HNK-1-positive sTM-myc was treated with peptide *N*-glycosidase F (PNGase F) to remove the *N*-linked glycans. The molecular weight of sTM-myc was slightly decreased, but HNK-1 immunoreactivity was unchanged after the PNGase F treatment (Fig. 1C), indicating that TM carries *N*-linked glycans but the HNK-1 epitope does not attach to it. Considering our previous finding that the linkage tetrasaccharide (GlcA $\beta$ 1-3Gal $\beta$ 1-3Gal $\beta$ 1-4Xyl) is the only oligosaccharide detected on  $\alpha$ -TM expressed in CHO-K1 cells and HNK-1ST transfers a sulfate group to the C-3 position of the non-reducing terminal GlcA, HNK-1ST appeared to be involved in the biosynthesis of the GAG-linkage-type HNK-1 epitope (HSO<sub>3</sub>-3GlcA $\beta$ 1-3Gal $\beta$ 1-3Gal $\beta$ 1-4Xyl) found in human urine [17].

#### 3.2. The sulfotransferase activity of HNK-1ST is required for regulating the attachment of the CS chain

Next, to determine the requirement of the enzymatic activity of HNK-1ST to arrest the initiation



of the CS chain, we generated an activity-depleted mutant of HNK-1ST, which harbors a mutation of Arg<sup>189</sup> to Ala (R189A-EGFP). This mutant is reported to show almost no activity due to impaired binding to the donor substrate, 3'-phosphoadenosine 5'-phosphosulfate (PAPS) [25]. When co-expressed with R189A-EGFP, sTM-myc contained a comparable amount of the  $\beta$ -form to that expressed alone and did not exhibit HNK-1 immunoreactivity on its  $\alpha$ -form (Fig. 2). This result indicates that the regulatory function of HNK-1ST is achieved by the transfer of a sulfate group to the C-3 position of GlcA. Taken together, these results suggest that the sulfation of terminal GlcA in the GAG-linkage tetrasaccharide by HNK-1ST negatively regulates the CS chain on TM.

### *3.3. HNK-1ST controls the anti-coagulant activity of TM by regulating the attachment of the CS chain*

TM is expressed on the endothelial cell surface, and plays a pivotal role in anti-coagulation, including the inhibition of the procoagulant activity of thrombin [10]. Since the anti-coagulant activity of TM is highly dependent on its CS chain [10, 12], we examined whether HNK-1ST is able to control the function of TM by regulating the attachment of the CS chain. To measure anti-coagulant activity, we purified the recombinant sTM-myc from the culture medium of CHO-K1 cells with or without ST-EGFP co-expression. The purity of the recombinant protein was confirmed by CBB staining and Western blotting (Fig. 3A, B). Consistent with the data shown in Fig. 1, co-expression of HNK-1ST resulted in a reduction in  $\beta$ -TM and the appearance of HNK-1-immunoreactivity (Fig. 3A, B). Then, a thrombin-dependent clotting assay was performed to evaluate the anti-coagulant activity of purified sTM-myc. sTM-myc (TM in Fig. 3C), which was not co-expressed with ST-EGFP, caused a dose-dependent delay in the clotting time, while the removal of the CS chain by the pre-treatment with CSase ABC greatly abrogated the anti-coagulant activity of sTM-myc (TM(CSase) in Fig. 3C). Then, we employed the same assay using sTM-myc co-expressed with ST-EGFP (TM+HNK-1ST). TM+HNK-1ST showed almost no inhibition of thrombin-induced clotting, similar to the CSase-treated sTM-myc (Fig. 3C). These results suggest that TM is scarcely modified by the CS chain in the presence of HNK-1ST, leading to the loss of TM function.

### *3.4. The terminal 3-O-sulfation of the linkage tetrasaccharide has an inhibitory function in the initiation of CS biosynthesis*

The initiation and elongation of CS chains are conducted by an enzyme complex of chondroitin

synthase (ChSy-1 to 3) and chondroitin polymerizing factor (ChPF) [22, 26]. Therefore, to demonstrate that the terminal sulfate group transferred by HNK-1ST to the linkage tetrasaccharide indeed acts as a stop signal that inhibits the addition of CS, we measured the chondroitin-polymerizing activity of ChSy/ChPF against sTM-myc described in Fig. 3. When sTM-myc expressed without ST-EGFP (TM in Table 1) was used as an acceptor substrate, significant polymerization by ChSy/ChPF (17.8 pmol/mg/h) was observed (Table 1). However, we could not detect any activity against sTM-myc co-expressed with ST-EGFP (TM+HNK-1ST in Table 1), indicating that the sulfated linkage region tetrasaccharide is not utilized as a substrate for CS-polymerizing enzymes.

In this report, we demonstrated that HNK-1ST synthesizes a HNK-1 antibody-reactive epitope on  $\alpha$ -TM and suppresses the expression of  $\beta$ -TM in an activity-dependent manner. We also produced evidence that  $\alpha$ -TM bearing this epitope is not utilized as a substrate for CS-polymerizing enzymes. We did not precisely determine the structure of the HNK-1-reactive epitope but the sulfate group transferred by HNK-1ST is presumably expressed on the terminal GlcA at the C-3 position of the GAG-linkage region. The reason for this is that HNK-1ST is known to transfer a sulfate group from PAPS to the C-3 position of the non-reducing terminal GlcA residue and the resultant epitope is recognized by HNK-1 antibody [27]. Furthermore, Hashiguchi *et al.* have directly demonstrated the sulfotransferase activity of HNK-1ST toward the GlcA of the GAG-linkage region with an *in vitro* enzymatic assay system using chemically synthesized substrates. These results suggest that HNK-1ST forms an enzyme complex with GlcAT-I and regulates the expression of the CS chain of TM to control anti-coagulation.

Since 3-*O*-sulfation suppressed the assembly of CS, this sulfate group might be used as an inhibitory signal to control the function of PGs through the modification of GAGs. At the initiation step of the polymerization of CS chains, the first GalNAc is transferred to the C-4 position of the terminal GlcA of the linkage region tetrasaccharide [1]. Therefore, the 3-*O*-sulfation by HNK-1ST does not directly compete with the transfer of the first GalNAc, and there might be an underlying mechanism by which the sulfate group inhibits the initial GalNAc-transfer, for example, steric hindrance. Although we have shown the effect of 3-*O*-sulfation on the attachment of CS chains, its influence on HS synthesis is unclear. Since the first GlcNAc of HS chains is also transferred to the C-4 position of the terminal GlcA, it is possible that the 3-*O*-sulfate exerts a similar inhibitory effect on the assembly of HS. However, the linkage tetrasaccharide containing 3-*O*-sulfated GlcA on HS-bearing PGs is still to be identified.

HNK-1ST is generally known as a sulfotransferase involved in the biosynthesis of a HNK-1 carbohydrate epitope mainly expressed on *N*-glycans in the nervous system. The expression of the HNK-1 carbohydrate is predominantly restricted to the nervous tissue due to limited distributions of GlcAT-P and GlcAT-S [28, 29]. HNK-1ST, however, is more ubiquitous than GlcAT-P or GlcAT-S [27, 30], supporting our idea that HNK-1ST contributes to the regulation of GAG attachment, a second role different from that in the biosynthesis of the HNK-1 epitope expressed on *N*-glycans.

The 3-*O*-sulfated tetrasaccharide structure has been found in  $\alpha$ -TM purified from human urine and serum [17, 20]. In this study, we demonstrated that the sulfated structure is biosynthesized by HNK-1ST and is not utilized as a substrate for CS-polymerizing enzymes. These lines of evidence indicate the possibility that the CS-modulating function of HNK-1ST is employed to maintain the homeostasis of the blood circulation system *in vivo*. In CHO-K1 cells, however, a significant amount of  $\alpha$ -TM bearing un-sulfated tetrasaccharide linkage structure was expressed even without HNK-1ST transfection (Fig. 1B, and [11]), indicating the presence of another regulatory mechanism of part-time PGs. Since the CS of TM plays a crucial role in its anti-coagulant activities, it is of great interest to clarify the overall sorting mechanism for part-time PGs.

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## References

- [1] K. Sugahara, H. Kitagawa, Recent advances in the study of the biosynthesis and functions of sulfated glycosaminoglycans, *Curr. Opin. Struct. Biol.* 10 (2000) 518-527.
- [2] C. Götting, J. Kuhn, R. Zahn, T. Brinkmann, K. Kleesiek, Molecular Cloning and Expression of Human UDP-D-Xylose:Proteoglycan Core Protein  $\beta$ -D-Xylosyltransferase and its First Isoform XT-II, *J. Mol. Biol.* 304 (2000) 517-528.
- [3] T. Okajima, K. Yoshida, T. Kondo, K. Furukawa, Human homolog of *Caenorhabditis elegans sqv-3* gene is galactosyltransferase I involved in the biosynthesis of the glycosaminoglycan-protein linkage region of proteoglycans, *J. Biol. Chem.* 274 (1999) 22915-22918.
- [4] R. Almeida, S.B. Levery, U. Mandel, H. Kresse, T. Schwientek, E.P. Bennett, H. Clausen, Cloning and expression of a proteoglycan UDP-galactose: $\beta$ -xylose  $\beta$ 1,4-galactosyl-transferase I. A seventh member of the human  $\beta$ 4-galactosyltransferase gene family, *J. Biol. Chem.* 274 (1999) 26165-26171.
- [5] X. Bai, D. Zhou, J.R. Brown, B.E. Crawford, T. Hennet, J.D. Esko, Biosynthesis of the linkage region of glycosaminoglycans: cloning and activity of galactosyltransferase II, the sixth member of the beta 1,3-galactosyltransferase family (beta 3GalT6), *J. Biol. Chem.* 276 (2001) 48189-48195.
- [6] H. Kitagawa, Y. Tone, J. Tamura, K.W. Neumann, T. Ogawa, S. Oka, T. Kawasaki, K. Sugahara, Molecular cloning and expression of glucuronyltransferase I involved in the biosynthesis of the glycosaminoglycan-protein linkage region of proteoglycans, *J. Biol. Chem.* 273 (1998) 6615-6618.
- [7] K. Sugahara, T. Mikami, T. Uyama, S. Mizuguchi, K. Nomura, H. Kitagawa, Recent advances in the structural biology of chondroitin sulfate and dermatan sulfate, *Curr. Opin. Struct. Biol.* 13 (2003) 612-620.
- [8] N. Perrimon, M. Bernfield, Specificities of heparan sulphate proteoglycans in developmental

processes, *Nature* 404 (2000) 725-728.

[9] L.Å. Fransson, Structure and function of cell-associated proteoglycans, *Trends Biochem. Sci.* 12 (1987) 406-411.

[10] K.T. Preissner, T. Koyama, D. Müller, J. Tschopp, G. Müller-Berghaus, Domain structure of the endothelial cell receptor thrombomodulin as deduced from modulation of its anticoagulant functions. Evidence for a glycosaminoglycan-dependent secondary binding site for thrombin, *J. Biol. Chem.* 265 (1990) 4915-4922.

[11] S. Nakanaka, H. Kitagawa, K. Sugahara, Demonstration of the immature glycosaminoglycan tetrasaccharide sequence GlcA $\beta$ 1-3Gal $\beta$ 1-3Gal $\beta$ 1-4Xyl on recombinant soluble human  $\alpha$ -thrombomodulin. A possible mechanism generating 'part-time' proteoglycans, *J. Biol. Chem.* 273 (1998) 33728-33734.

[12] K. Nawa, K. Sakano, H. Fujiwara, Y. Sato, N. Sugiyama, T. Teruuchi, M. Iwamoto, Y. Marumoto, Presence and function of chondroitin-4-sulfate on recombinant human soluble thrombomodulin, *Biochem. Biophys. Res. Commun.* 171 (1990) 729-737.

[13] S. Yamamoto, S. Oka, M. Inoue, M. Shimuta, T. Manabe, H. Takahashi, M. Miyamoto, M. Asano, J. Sakagami, K. Sudo, Y. Iwakura, K. Ono, T. Kawasaki, Mice deficient in nervous system-specific carbohydrate epitope HNK-1 exhibit impaired synaptic plasticity and spatial learning, *J. Biol. Chem.* 277 (2002) 27227-27231.

[14] I. Morita, S. Kakuda, Y. Takeuchi, T. Kawasaki, S. Oka, HNK-1 (human natural killer-1) glyco-epitope is essential for normal spine morphogenesis in developing hippocampal neurons, *Neuroscience* 164 (2009) 1685-1694.

[15] I. Morita, S. Kakuda, Y. Takeuchi, S. Itoh, N. Kawasaki, Y. Kizuka, T. Kawasaki, S. Oka, HNK-1 glyco-epitope regulates the stability of the glutamate receptor subunit GluR2 on the neuronal cell surface, *J. Biol. Chem.* 284 (2009) 30209-30217.

[16] Y. Kizuka, T. Matsui, H. Takematsu, Y. Kozutsumi, T. Kawasaki, S. Oka, Physical and

functional association of glucuronyltransferases and sulfotransferase involved in HNK-1 biosynthesis, *J. Biol. Chem.* 281 (2006) 13644-13651.

[17] H. Wakabayashi, S. Natsuka, T. Mega, N. Otsuki, M. Isaji, M. Naotsuka, S. Koyama, T. Kanamori, K. Sakai, S. Hase, Novel proteoglycan linkage tetrasaccharides of human urinary soluble thrombomodulin,  $\text{SO}_4\text{-3GlcA}\beta\text{1-3Gal}\beta\text{1-3(+/-Sia}\alpha\text{2-6)Gal}\beta\text{1-4Xyl}$ , *J. Biol. Chem.* 274 (1999) 5436-5442.

[18] Y. Tone, L.C. Pedersen, T. Yamamoto, T. Izumikawa, H. Kitagawa, J. Nishihara, J. Tamura, M. Negishi, K. Sugahara, 2-*O*-phosphorylation of xylose and 6-*O*-sulfation of galactose in the protein linkage region of glycosaminoglycans influence the glucuronyltransferase-I activity involved in the linkage region synthesis, *J. Biol. Chem.* 283 (2008) 16801-16807.

[19] T. Koike, T. Izumikawa, J. Tamura, H. Kitagawa, FAM20B is a kinase that phosphorylates xylose in the glycosaminoglycan-protein linkage region, *Biochem. J.* 421 (2009) 157-162.

[20] T. Hashiguchi, S. Mizumoto, Y. Nishimura, J.I. Tamura, S. Yamada, K. Sugahara, Involvement of HNK-1 sulfotransferase in the biosynthesis of the GlcUA(3-*O*-sulfate)-Gal-Gal-Xyl tetrasaccharide found in  $\alpha$ -thrombomodulin from human urine, *J. Biol. Chem.* 286 (2011) 33003-33011.

[21] C.T. Esmon, N.L. Esmon, K.W. Harris, Complex formation between thrombin and thrombomodulin inhibits both thrombin-catalyzed fibrin formation and factor V activation, *J. Biol. Chem.* 257 (1982) 7944-7947.

[22] H. Kitagawa, T. Izumikawa, T. Uyama, K. Sugahara, Molecular cloning of a chondroitin polymerizing factor that cooperates with chondroitin synthase for chondroitin polymerization, *J. Biol. Chem.* 278 (2003) 23666-23671.

[23] H. Kitagawa, K. Tsutsumi, M. Ujikawa, F. Goto, J. Tamura, K. Neumann, T. Ogawa, K. Sugahara, Regulation of chondroitin sulfate biosynthesis by specific sulfation: acceptor specificity of serum  $\beta$ -GalNAc transferase revealed by structurally defined oligosaccharides, *Glycobiology* 7 (1997) 531-537.

- [24] H. Kitagawa, K. Tsuchida, M. Ujikawa, K. Sugahara, Detection and characterization of UDP-GalNAc: chondroitin *N*-acetylgalactosaminyltransferase in bovine serum using a simple assay method, *J. Biochem.* 117 (1995) 1083-1087.
- [25] E. Ong, J.C. Yeh, Y. Ding, O. Hindsgaul, L.C. Pedersen, M. Negishi, M. Fukuda, Structure and function of HNK-1 sulfotransferase. Identification of donor and acceptor binding sites by site-directed mutagenesis, *J. Biol. Chem.* 274 (1999) 25608-25612.
- [26] T. Izumikawa, T. Koike, S. Shiozawa, K. Sugahara, J. Tamura, H. Kitagawa, Identification of chondroitin sulfate glucuronyltransferase as chondroitin synthase-3 involved in chondroitin polymerization: chondroitin polymerization is achieved by multiple enzyme complexes consisting of chondroitin synthase family members, *J. Biol. Chem.* 283 (2008) 11396-11406.
- [27] E. Ong, J.C. Yeh, Y. Ding, O. Hindsgaul, M. Fukuda, Expression cloning of a human sulfotransferase that directs the synthesis of the HNK-1 glycan on the neural cell adhesion molecule and glycolipids, *J. Biol. Chem.* 273 (1998) 5190-5195.
- [28] K. Terayama, S. Oka, T. Seiki, Y. Miki, A. Nakamura, Y. Kozutsumi, K. Takio, T. Kawasaki, Cloning and functional expression of a novel glucuronyltransferase involved in the biosynthesis of the carbohydrate epitope HNK-1, *Proc. Natl. Acad. Sci. USA* 94 (1997) 6093-6098.
- [29] T. Seiki, S. Oka, K. Terayama, K. Imiya, T. Kawasaki, Molecular cloning and expression of a second glucuronyltransferase involved in the biosynthesis of the HNK-1 carbohydrate epitope, *Biochem. Biophys. Res. Commun.* 255 (1999) 182-187.
- [30] H. Tagawa, Y. Kizuka, T. Ikeda, S. Itoh, N. Kawasaki, H. Kurihara, M.L. Onozato, A. Tojo, T. Sakai, T. Kawasaki, S. Oka, A non-sulfated form of the HNK-1 carbohydrate is expressed in mouse kidney, *J. Biol. Chem.* 280 (2005) 23876-23883.

## Figure legends

### Figure 1. Effect of HNK-1ST on the expression of the HNK-1 epitope and CS chain on TM.

(A) sTM-myc with or without HNK-1ST-EGFP (ST-EGFP) was transiently expressed in CHO-K1 cells. The secreted sTM-myc was pulled down from the culture medium and subjected to Western blotting using anti-myc and HNK-1 mAb (*medium*). The two glycoforms of TM ( $\alpha$ - and  $\beta$ -TM) were detected (*arrowheads*). The CHO-K1 cell lysate was analyzed for HNK-1ST-EGFP expression (*cell lysate*). (B) sTM-myc was treated with chondroitinase ABC (CSase ABC) to remove the CS moiety and Western blotted with anti-myc mAb. (C) sTM-myc was prepared from HNK-1ST-EGFP co-expressing cells and treated with peptide *N*-glycosidase F (PNGase F), and subjected to Western blotting with anti-myc and HNK-1 mAb.

### Figure 2. Requirement of the sulfotransferase activity of HNK-1ST for the suppression of

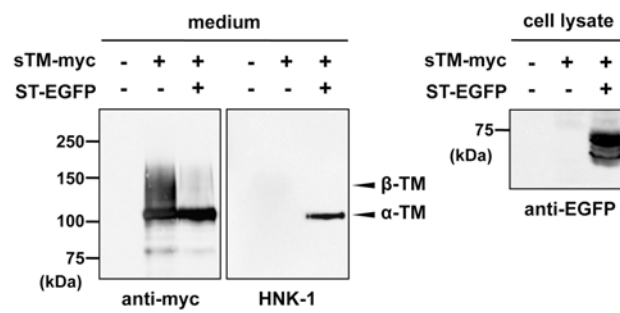
**CS chain on TM.** sTM-myc was co-transfected with ST-EGFP or the R189A mutant of HNK-1ST (R189A-EGFP). The secreted sTM-myc was pulled down and subjected to Western blotting using anti-myc and HNK-1 mAb (*medium*). The expression of HNK-1ST and HNK-1ST (R189A) was confirmed by Western blotting of the cell lysate (*cell lysate*).

### Figure 3. The effect of HNK-1ST on the anti-coagulant activity of TM.

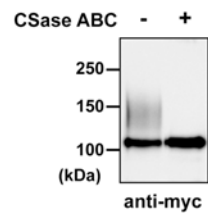
(A, B) sTM-myc expressed in the absence or presence of ST-EGFP was purified from the culture medium of CHO-K1 cells using a HisTrap column. The culture medium (*charge*), the pass-through fraction (*pass*), and the eluate were analyzed by CBB staining (A) and Western blotting using anti-myc (B). The HNK-1 expression was also assessed using the eluates (B). (C) The purified sTM-myc was subjected to the thrombin-dependent clotting assay. sTM-myc (TM, *open circle*), sTM-myc pre-treated with CSase ABC (TM(CSase), *filled circle*), and sTM-myc co-expressed with HNK-1ST (TM+HNK-1ST, *triangle*) were mixed with thrombin at different molar ratios as indicated and clotting time was measured.



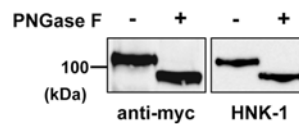
**A**

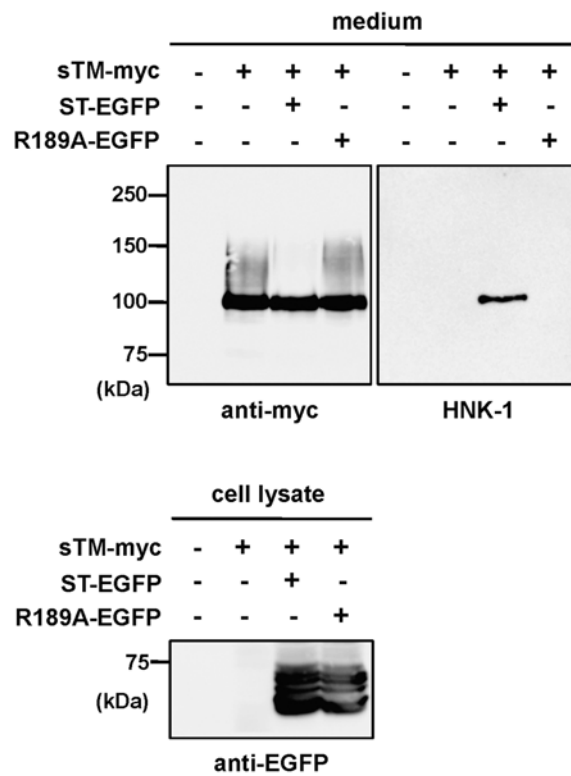


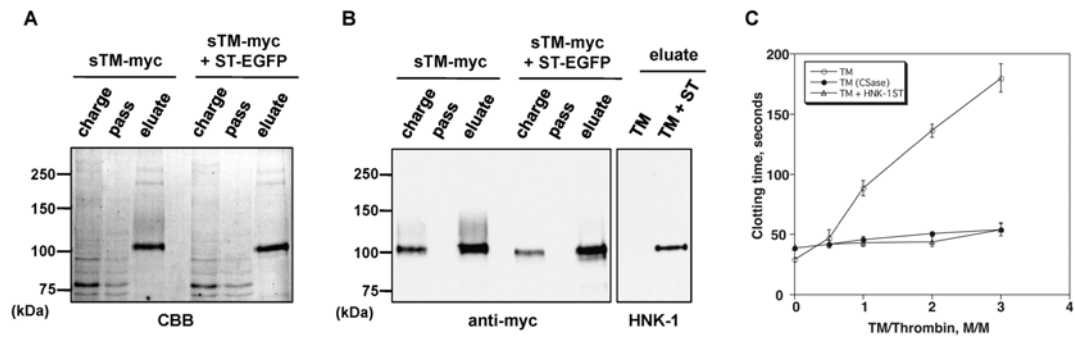
**B**



**C**







**Table 1 Chondroitin polymerization on HNK-1-positive sTM-myc**

Acceptor substrate	Polymerization activity
	<i>pmol/mg/h</i>
TM (0.1 nmol)	17.8
TM+HNK-1ST (0.1 nmol)	ND <sup>a</sup>

<sup>a</sup>ND, not detected (<0.01 pmol/mg/h)